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(71) Applicant *(for all designated States except US)*: AGRICUL-TURAL & FOOD RESEARCH COUNCIL [GB/GB]; Shinfield, Reading, Bekshire RG2 9AT (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: GASSON, Michael, John [GB/GB]; Old Ash Farm, Stone Lane, Bintree, Dereham, Norfolk NR20 5NA (GB).

(74) Agent: BASSETT, Richard, Simon; Eric Potter & Clarkson, 14 Oxford Street, Nottingham NG1 5BP (GB).

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(54) Title: USES OF VIRAL ENZYMES

(57) Abstract

The lysin from a Lactoccus (preferably prolate-headed) bacteriophage is used to lyse bacterial starter cultures during cheese-making. Such bacteriophages include ØvML3. In addition, the OvML3 Lysin has been characterised and a coding sequence for it has been cloned.

USES OF VIRAL ENZYMES

The bacteriophage ϕvML3. which uses Streptococcus lactis ML3 as host, produces a bacteriophage lysin that causes distinct "halos" around viral plaques. This lysin is responsible for cell-wall degradation and lysis of the host cells. Streptococcus lactis has recently been re-named as Lactococcus lactis subsp. lactis.

The bacteriophage lysin of ϕvML3 has been partially purified and its ability to lyse bacteria other than Lactococcus lactis subsp. lactis ML3 has been investigated previously. The lysin was found to lyse strains of Lactococcus lactis subsp. lactis and subsp. cremoris and to have a weak effect on group D streptococci. Other bacterial genera tested were not affected [Oram, J.D. & Reiter, B. (1965) J. Gen. Microbiol. 40, 57-70; Reiter, B. & Oram, J.D. (1963) J. Gen. Microbiol. 32, 29-32].

The lysis of cheese-making bacteria by bacterio-phages, or by other means, has previously been found to be a problem. We have now found that a substantially pure lysin enzyme can actually be used to advantage in the manufacture of cheese.

A first aspect of the invention provides a substantially pure preparation of the ΦvML3 lysin or a variant thereof.

A second aspect of the invention provides a formulation comprising the lysin of a Lactoccus bacteriophage or a variant of such a lysin and suitable for addition to cheese or to a cheese precursor mixture. A formulation is suitable for addition to cheese or to a precursor if it does not immediately or later render the cheese unfit for consumption or prevent the formation of consumable cheese from the precursor mixture. Suitably, the formulation is substantially free, preferably completely free, of viable bacteriophage. Preferably, the bacteriophage is a prolate-headed bacteriophage.

A third aspect of the invention provides a coding sequence for such a lysin, in isolation from the genes which would normally lie immediately upstream or immediately downstream of that gene in the associated bacteriophage, or a variant of such a coding sequence. A conservative variant is one in which the DNA sequence is different from that found naturally but, because of the degeneracy of the genetic code, encodes the same polypeptide fragments as does the nature-identical

sequence. Other variants include coding sequences encoding polypeptides which have slightly different amino acid sequences from the lysin but which retain at least 10%, preferably at least 50%, 90%, 95% or 99% of the bacterial cell wall-degrading activity of the said lysin. Such activity may be measured by observing a change in optical density of a suspension of cells, for example Lactoccus lactis subsp. lactis, exposed to the lysin. Such variant polypeptides may include those which are slightly longer or shorter than the lysin or which have conservative amino acid substitutions For example, a serine may be substituted for a threonine and vice versa and a glutamine may be substituted for an asparagine and vice versa. Generally, substitutions, deletions and additions at the N-terminus have less effect on function than at the C-terminus.

A fourth aspect provides a coding sequence as above in an expression vehicle suitable for transformation of a microbial host. The expression vehicle may have a control region for the coding sequence. the control region including a start codon upstream of the gene and the gene being in proper reading frame relative to the start codon or the expression vehicle may provide for insertion of the coding sequence into an endogenous

heritable unit so that the coding sequence is under the
control of such a control region therein. The control
region may comprise the natural viral control region, a
lactic streptococcal control region (such as the lactose
operon) or other control regions which control,
preferably inducibly, expression of the gene in the
microbial host. For example, in E.coli one might use the
lac control elements or those of the arabinose, colicine
E1, galactose, alkaline phosphatase, tryptophan or lambda
operons. In yeast (e.g.Saccharomyces cerevisiae), one
might use the promoters for phosphoglycerokinase or for
galactose.

Thus, a fifth aspect of the invention provides a
microbial host transformed with such an expression
vehicle and capable of expressing the lysin coding
sequence. The microbial host may be any microorganism
which is found to express the said lysin gene and may be
a bacterium (such as E. coli or Bacillus subtilis), a
fungus (such as S. cerevisiae or Aspergillus nidulans), a
plant cell, a plant protoplast, or an animal cell.
Preferably the host is B.subtilis or Lactobacillus.

Provided that the cell wall of the host is not
itself degraded by the lysin, then the lysin-secreting
transformed host may be useful in suppressing populations
of bacteria which are susceptible to lysis by the lysin.

A sixth aspect provides a polypeptide derived from
the expression of the said lysin coding sequence in a
suitable microbial host transformed with such an
expression vehicle. The invention also encompasses
cloning vehicles comprising such coding sequences.

A seventh aspect provides a process for at least
partially isolating the lysin gene from a prolate-headed
bacteriophage by preparing from the DNA thereof the
restriction fragment EcoRV-EcoRI of Figure 7 or a
corresponding fragment thereof, preferably in isolation
from neighbouring bacteriophage sequences. (The EcoRI
restriction site is actually created artificially by
adding an EcoRI linker to an AluI site). The lysin gene
of bacteriophages other than OVML3 can generally be
obtained by identifying the bacterial cell wall lysing
proteolytic enzyme thereof, sequencing it, creating a
mRNA probe and isolating the gene, all in known ways.

An eighth aspect of the invention provides a method of making cheese comprising lysing the bacterial starter culture at a predetermined time during the preparation of the cheese by the addition of a Lactococcus bacteriophage lysin. Suitable starter culture bacteria include Lactococcus lactis subsp. lactis and cremoris. Preferably, the lysin is added at the end of the dairy fermentation such that the starter cells of the fermentation are lysed, thus releasing the intracellular enzymes. Some of these enzymes can then cause the mature flavour of cheese to develop and thus a method in accordance with the invention may be used to accelerate the maturation of the cheese. The lysin, at least in the quantities necessary, does not render the resulting cheese unfit for human consumption and may readily be removed from the cheese-making container or destroyed before the next starter culture is introduced.

A ninth aspect of the invention provides cheese made by such a process.

Prolate-headed bacteriophages include the following bacteriophages of Lactococcus lactis:

Bacteriophage	Subspecies
c2	lactis c2
c6a	lactis c6 or c10
c6B	lactis c6 or c10

c6D	lactis c6 or c10
C10I	lactis c10
C10w	lactis c10
drc1	lactis DRC1
643	lactis C8

Preferably, in all of the aspects of the invention discussed above, the lysin is the lysin from the bacteriophage ϕvML3. The bacteriophage is well known but, to avoid any possible doubt, we have deposited it at the National Collections of Industrial and Marine Bacteria, Aberdeen under the terms of the Budapest Treaty. The date of deposit was 5th July 1989 and the accession number is NCIMB 40160.

We have similarly deposited the natural host of ϕvML3, namely Lactococcus lactis subsp. lactis ML3, on 5th July 1989 as NCIMB 40161.

A person skilled in the art will readily be able to determine the most appropriate conditions of use of the lysin, for example the time of introduction of the-lysin, the amount to be introduced, the nature of the composition being introduced and so on. These may vary according to the type of cheese being made. Generally,

however, the lysin is added as a small volume (preferably no more than 5% or 1% of the volume of the cheese culture) of a sterile solution to give a concentration in the cheese fermentation culture of between 0.01 μg/l and 1500 mg/l. Or, the lysin may be encapsulated in liposomes (as in Kirby et al, (1987) Int. J. Food Sci. Tech. 22, 355-375) and added at the outset for delayed release.

Preferred embodiments of the invention will now be described by way of example and with reference to the accompanying drawings, in which:-

Figure 1 (on two sheets) is a scheme of the stages in gene cloning used to isolate the ØvML3 lysin gene;

Figure 2 is a photograph of vector λ.gt10 plaques and clones which express the lysin gene of bacteriophage ØvM13;

Figure 3 is a photograph of an E. coli strain carrying the clone pFI106 which expresses bacteriophage ØvML3 lysin;

Figure 4 is a restriction and deletion map of a fragment of bacteriophage ØvML3 encoding the lysin gene;

Figure 5 is a fluorograph of proteins expressed by pUC13 plasmid vector DNA, by plasmid pFI106 which expresses lysin and by plasmid pFI115 which does not express lysin;

Figure 6 is a scheme showing the DNA sequencing strategy used; and

Figure 7 shows the total sequence of a 1.2 kilobase fragment of bacteriophage φvML3 DNA encoding the lysin gene which was cloned in λ.gt10-lysin 4, including the DNA sequence and deduced amino acid sequence for the bacteriophage lysin gene.

EXAMPLE 1 : CLONING OF LYSIN GENE ETC.

Preparation of bacteriophage φvML3 DNA

High titre bacteriophage OvML3 lysates were prepared by inoculating 500ml of lactose M17 broth (Terzaghi & Sandine, 1975) with 2% of an overnight lactose M17 broth culture of Lactococcus lactis subsp. lactis ML3. This is freely available from the National Collection of Food Bacteria, Shinfield, Reading, Berks., U.K. The culture was grown to an O.D. 600 of 1.00 ± 0.05 and 5ml of 0.05M calcium borogluconate and 10ml of φvML3 bacteriophage

10

lysate with a concentration of 3.3×10^{10} plaque-forming units per ml were added. φvML3 is also freely available from NCFB. Incubation was continued until complete lysis occurred and no further fall in optical density was observed (the optical density fell to an O.D. 600 of 0.34). Cell debris was removed by centrifugation at 8000 x g for 15 minutes.

Bacteriophage were concentrated by centrifugation of the lysate at 125,000 x g for 3 hours. The supernatant was discarded and the bacteriophage pellet was resuspended by storage overnight at 4°C in 8ml of a buffer consisting of 0.01M Tris hydrochloride, 0.1mM EDTA, and 1mM magnesium sulphate, pH 8.0.

The bacteriophage suspension was centrifuged at 1,600 x g for 5 minutes and 4ml of the supernatant was layered onto a stepped gradient prepared from 2ml aliquots of caesium chloride solutions in buffer (as above), having specific gravities of 1.6, 1.5, 1.4 and 1.3. Gradients were centrifuged at 92,000 x g for 2 hours at 5°C in a swing-out centrifuge rotor. The band of bacteriophage formed was removed from the gradient with a small hypodermic syringe in a volume of approximately 0.5 ml and mixed with an equal volume of saturated caesium

chloride solution in buffer (as above). This was placed at the bottom of a similar stepped gradient and centrifuged as before and once again the bacteriophage band was removed and placed in a 1.5ml Eppendorf tube.

The high titre bacteriophage suspension was lysed to release DNA by replacing the top of the Eppendorf tube with dialysis membrane, held in place with a tight rubber band, and performing dialysis for 18 hours at room temperature against 50% formamide in a buffer consisting of 0.1M Tris hydrochloride, 0.01M EDTA, pH 8.5. Formamide was removed by dialysis for 24 hours at 4°C against a buffer consisting of 0.1M Tris hydrochloride, 0.1mM EDTA, pH 7.5. The resultant bacteriophage DNA was purified by caesium chloride-ethidium bromide equilibrium density centrifugation for 60 hours at 125,000 x g. The DNA band was visualised under ultra-violet light and removed with a small hypodermic syringe. Ethidium bromide was extracted with caesium chloride saturated iso-amyl alcohol and caesium chloride was removed by dialysis for 24 hours at 4°C against a buffer consisting of 0.1M Tris hydrochloride, 0.1mM EDTA, pH 7.5.

Cloning the Bacteriophage φvML3 lysin gene

The bacteriophage φvML3 DNA was a 23.3 kilobase
molecule with 3 recognition sites for restriction
endonuclease EcoRI. A restriction map for the bacterio-
phage is shown in Figure 1. Bacteriophage ϕvML3 DNA was
partially digested with restriction endonuclease AluI in
a reaction buffer to produce random blunt-ended DNA
fragments. These were separated by electrophoresis at 4°C
in low-melting-point agarose (Sigma) and that region of
separated DNA containing fragments in the size range 0.5
to 4.0 kilobases was cut from the gel. The agarose block
was mixed with 5 volumes of a buffer consisting of 20mM
Tris hydrochloride, 1mM EDTA, pH 8.0 and melted by
heating to 65°C for 5 minutes. At room temperature the
DNA and agarose solution was successively extracted with
equal volumes of phenol, phenol-chloroform and chloroform
and the DNA was recovered by ethanol precipitation and
resuspension in TE buffer. Eight base-pair phosphorylated
linkers with the DNA sequence 5'-GGAATTCC-3' (Pharmacia),
which includes the recognition site for restriction
endonuclease EcoRI, were ligated to the purified blunt-
ended fragments of bacteriophage ϕvML3 DNA. The ligation
was performed overnight at 15°C in a reaction mixture
consisting of blunt-ended φvML3 DNA fragments, linker DNA

and 5 units of DNA ligase enzyme (Boehringer Mannheim) in 50ul of a buffer consisting of 70mM Tris hydrochloride, 7mM magnesium chloride, 0.07mM ATP, pH 7.5. The reaction was stopped by incubation for 10 minutes at 65°C and the linkers were trimmed by digestion at 37°C for 2 hours with restriction endonuclease EcoRI in 100ul of a buffer consisting of 100mM Tris hydrochloride, 50mM sodium chloride, 10mM magnesium chloride, pH 7.5. The reaction was stopped by phenol extraction and the fragments of bacteriophage OvML3 were purified by chromatography over a NENSORB 20 column (DuPont).

Purified, EcoRI-digested and dephosphorylated DNA arms of bacteriophage lambda vector λ.gt10 were purchased from Northumbria Biologicals Ltd and ligated with pure DNA fragments of ϕvML3 DNA by incubation overnight at 4°C with 1 unit of DNA ligase per 10ul in a reaction buffer containing 50mM Tris hydrochloride, 7mM magnesium chloride, 1mM dithiothreitol, 1mM ATP, pH8. Ligated DNA was packaged in vitro into lambda bacteriophage using crude cell-extracts prepared as described by Scalenghe et al. (1981) and Hohn (1979). Freeze-thaw lysates (FTL) were prepared from E.coli strain BHB2688 and sonic extracts from E. coli strain BHB 2690. The in vitro packaging reaction was carried out by the sequential

addition of 350ul buffer consisting of 20mM Tris hydrochloride, 3mM magnesium chloride, 0.05% ß-mercapto-ethanol, 1uM EDTA, pH 8.0; 50ul of ligated DNA; 50ul of a buffer consisting of 30mM spermine tri-hydrochloride pH 7.0, 30mM putrescine-dihydrochloride pH 7.0, 18mM magnesium chloride, 15mM ATP, 1.2% ß-mercaptoethanol; 175ul of sonic extract (SE); 250ul freeze-thaw extract (FTL). The mixture was incubated for 90 minutes at room temperature and 11.5ml of lambda buffer consisting of 6mM Tris hydrochloride, 10mM magnesium chloride, 100mM sodium chloride, 0.5mg/ml gelatine, pH 8.0 was added, together with 50ul of chloroform. The bacteriophage lambda suspension was plated on the E. coli host strain C600 hfl⁻ using NZY agar plates. Lambda plaques that expressed the bacteriophage ɸvML3 lysin gene were detected by overlaying with a suspension of Lactococcus lactis subsp. cremoris NCDO 1196 cells in 0.1M potassium phosphate buffer pH 6.7 with 0.7% agarose. Plates were incubated at 30°C for 6 hours and clones expressing bacteriophage OvML3 lysin produced a region of clearing above the lambda plaque as shown in Figure 2. A collection of 50 clones, each expressing lysin, were purified by single-plaque isolation and retained.

In Figure 2, plaques of λ.gt10 with random cloned fragments of bacteriophage ϕvML3 DNA are shown. The plaques are overlayed with <u>Lactoccus   lactis</u> subsp <u>cremoris</u> cells. Lambda clones which express a cloned lysin gene (arrowed) have produced a clear window in this lawn and appear dark, whereas other lambda plaques retain a cloudy appearance.

Characterisation of bacteriophage OvML3 lysin gene

One bacteriophage lambda clone that expressed the bacteriophage ϕvML3 lysin gene (λ.gt 10-lysin4) was characterised in detail. A large scale bacteriophage lambda lysate was prepared and DNA extracted as described above for bacteriophage ϕvML3. Digestion with restriction endonuclease <u>Eco</u>RI revealed the presence of a 1.2 kilobase DNA fragment from bacteriophage ϕvML3 which had cloned into the <u>Eco</u>RI site of lambda vector 2.gt 10.

This fragment was recloned into the EcoRI site of
the E. coli plasmid vector pUC13. Vector DNA and λgt10-
lysin 4 DNA were both digested with restriction endo-
nuclease EcoRI, using a buffer consisting of 100mM Tris
hydrochloride, 50mM sodium chloride, 10mM magnesium
chloride, pH 7.5. The reaction was stopped by phenol
extraction and a mixture of the cleaved DNAs was
incubated overnight at 15°C with 1 unit of DNA ligase in
a reaction buffer consisting of 50mM Tris - hydro-
chloride, 7 mM magnesium chloride, 1mM dithiothreitol,
1mM ATP, pH 8. Ligated DNA was transformed into competent
cells of E. coli strain TBI and ampicillin-resistant
colonies were selected on B agar containing IPTG and
"Bluogal" (BRL). Vectors with cloned DNA were identified
by their white colour. Their ability to produce bacterio-
phage φvML3 lysin was tested by overlaying colonies with
a suspension of Lactococcus lactis subscp. cremoris NCDO
1196 cells in 0.1M potassion phosphate buffer, pH 6.7,
with 0.7% agarose. Zones of clearing, as illustrated in
Figure 3, for one isolated clone were detected.   (In
Figure 3, colonies of Escherichia coli carrying plasmid
pFI 106 are overlayed with Lactococcus lactis subsp.
cremoris cells.   Expression of lysin causes a clear zone
of lysin in the cloudy lawn of Lactococcus cells.) The

plasmid from this clone named pFI106 was isolated and
analysed by restriction endonuclease mapping. It had the
structure shown in Figure 4.

In Figure 4, the independently determined
restriction map is confirmed by data derived from the DNA
sequence. (RI: EcoRI; M: MluI; RV: EcoRV; HII: HaeII;
Mb: MboII; D: DraI; H: HincII; Dd: DdeI; HIII: HaeIII; B:
BanI; C: CfoI; N: NarI; T: TaqI) The precise locations
of the lysin gene and ORF1 are deduced from the sequence
analysis. The broken line for ORF1 indicates its
possible extension upstream of methionine residues
detected by sequence analysis. In the deletion analysis,
regions of DNA retained in sub-clones are indicated by
solid bars. The presence or absence of lysin activity in
these constructs is indicated by + or -.

The 1.2 kilobase fragment of bacteriophage ϕvML3 DNA
that expressed lysin activity was recloned using the
strategy described above for vector pUC13 but into lactic
streptococcal vectors pCK1, pCK536 and pTG262. Clones
were selected by their resistance to chloramphenicol and
detected by their ability to produce lysin when overlaid
with Lactococcus lactis subsp. cremoris NCDO 1196 cells
as described above. Examples of constructed clones

consisting of lactic streptococcal vectors able to express the lysin gene of bacteriophage OvML3 are shown in Figure 1.

From pFI106 a series of deleted derivatives of the cloned 1.2 kilobase fragment of bacteriophage OvML3 DNA were made using restriction endonuclease digestion and either re-ligation or recloning into another vector. The details of construction and the structure of these derivatives are shown in Figure 4. All of the derivatives were tested for their ability to produce bacteriophage φvML3 lysin and the results are also shown in Figure 4. From the results it was possible to define the region of bacteriophage φvML3 that encodes the lysin gene. It is between coordinate 0.37 (DraI site) and coordinate 1.2 (EcoRI site) of the restriction endonuclease map shown in Figure 4. The region of DNA between coordinate 0.565 (HincII site) and coordinate 0.71 (HincII site) is essential for the expression of lysin and this region is deleted in derivative plasmid pFI115 as shown in Figure 4.

The proteins encoded by plasmid pFI106, which expresses the bacteriophage φvML3 lysin, and the derivative plasmid pFI115, which does not express lysin,

were compared using the <u>in vitro</u> transcription and
translation technique. Radioactively labelled [35]S
L-methionine was incorporated into proteins expressed by
these plasmid DNAs using the reagents and methodology of
a commercial kit (Amersham). Radio-labelled proteins that
were expressed were separated by SDS polyacrylamide gel
electrophoresis and detected by fluorography. As shown in
Figure 5, plasmid pFI106 expressed a protein of 23 kilo-
daltons which was not expressed by either the vector
pUC13 or by the deleted derivative plasmid pFI115. The
lysin enzyme of bacteriophage OvML3 is shown to be a
protein of 23 kilodaltons.

In Figure 5, the fluorograph is of radioactively
labelled proteins expressed by plasmid vectors and clones
during <u>in vitro</u> transcription/translation and separated
by SDS polyacrylamide gel electrophoresis. ^{35}S-labelled
proteins expressed by pAT153 control DNA (lane A); no DNA
(lane B); pUC19 vector DNA (lane C), pFI106 DNA which
expresses cloned lysin (lane D), pFI115 DNA in which part
of the lysin gene is deleted (lane E) are shown. Protein
molecular weight standards labelled with ^{14}C are shown to
the left.

Referring to Figure 6, the 1.2 kilobase EcoRI fragment containing the φvML3 lysin gene and derivative fragments were cloned into M13mp18. The clones were used as a source of DNA templates for DNA sequence determination by the Sanger dideoxy chain termination method. DNA sequences determined are indicated by the direction and extent of the arrows. Sequences indicated by boxed arrows were primed by synthetic oligonucleotides designed from already determined sequence data. These synthetic primers are also indicated in Fig. 6.

In addition to sequences determined by initiation from M13 DNA, using commercially available M13 primers, a number of synthetic primers were made from φvML3 DNA sequences that had already been determined. These are also shown in Figure 7. The complete DNA sequence for both strands of the 1.2 kilobase DNA fragment of bacteriophage φvML3 was determined. This is presented in Figure 7. The sequence was analysed using the Staden "Analseq" programme, which revealed an open reading frame consistent with the location of the lysin gene as revealed by deletion mapping and lysin expression determination (Figure 4) and with the molecular weight of the lysin enzyme determined by in vitro transcription and

translation of plasmids pFI106 and pFI115. The DNA
sequence for the lysin gene of bacteriophage OvML3 and
its deduced amino-acid sequence are shown in Figure 7.

Referring to Figure 7, restriction endonuclease sites
used in the construction of M13mp18 clones for sequence
determination are indicated. Solid lines show the
synthetic primers that were used in the sequencing
strategy (Fig.4). Primers 1 and 3 were as shown, whereas
primers 2, 4 and 5 were the complementary sequence. The
lysin structural gene is located between positions 593
and 1153. Its amino-acid sequence and that of ORF1 are
shown. For ORF1 in-frame methionine residues are boxed.
The possible lactic streptococcal promoter (position 550
to 580) and ribosome binding site (position 585) for the
lysin gene are also boxed.

EXAMPLE 2 : MODEL CHEESE-MAKING PROCESS

Starter cell lysis by cloned bacteriophage lysin

An E. coli strain carrying plasmid pFI106 was grown
to stationary phase in L-broth and washed and resuspended
in a one-tenth volume of a buffer consisting of 0.1M
potassium phosphate, pH 6.7. Cells were broken by four

30-second bursts of ultrasonication using an MSE ultra-
sonicator. Cell debris was removed by centrifugation for
30 minutes at 4°C and 20,000 x g.

The supernatant was used as a crude lysin
preparation. A negative control was prepared in the same
way from an E. coli strain carrying plasmid pFI115, which
does not express bacteriophage ϕvML3 lysin. Subspecies
cremoris cells suspended in a buffer consisting of 0.1M
potassium phosphate, pH 6.7, were rapidly lysed by the
crude lysin preparation: with lysin, the OD_{600} fell from
1.2 to 0.19 in 7 minutes, whereas without lysin the OD_{600}
fell only to about 1.19. A Subspecies cremoris cheese
starter strain was grown in milk for 6 hours in an
experiment that modelled a dairy fermentation. Crude
lysin preparation, a similar preparation from the
negative control and buffer were added to three separate
aliquots of the fermented milk and stored at 4°C. The
viable count of subspecies cremoris cells was monitored
and, as shown in Table 1 a rapid loss of viable cells due
to lysis was observed in the milk fermentation treated
with crude lysin preparation but not in the negative
control or buffer experiments.

Table 1

A 6-HOUR MILK FERMENTATION USING LACTOCOCCUS LACTIS

SUBSP. CREMORIS NCDO 1196 STARTER

(Starting Viable Count 3.45×10^9)

	Viable Count			% Viability		
	24h	48h	72h	24h	48h	72h
No addition	2.9×10^9	2.8×10^9	1.6×10^9	81	81	46
Extract of lysin-producing clone	1.5×10^6	1.5×10^6	1.3×10^5	0.04	0.01	0.04
Extract of lysin-negative control	3.0×10^9	3.3×10^9	1.5×10^9	87	96	44

EXAMPLE 3 : PREPARATION OF LYSIN COMPOSITION FOR CHEESE-MAKING

A bacterial culture which expresses the bacteriophage
lysin (e.g. E.coli carrying plasmid pFI106) is grown
overnight in broth. A culture (400ml) is centrifuged and
the cells resuspended in a one tenth volume of TE buffer.
Lysin is released from the cells, e.g. by chloroform

addition and centrifugation. The lysin-rich supernatant
is precipitated with 45% ammonium sulphate and the pellet
resuspended in 3ml of 0.1m potassium phosphate buffer
pH6.7 and passed through a PD10 column to desalt the
sample. Further purification was by F.P.L.C. using an
anion exchange column and a Sepharose 6 gel filtration
column.

EXAMPLE 4 : USE OF BACTERIOPHAGE LYSIN IN CHEDDAR
CHEESEMAKING

A conventional manufacturing process for cheddar cheese
is followed. After milling and salting, a preparation of
cloned bacteriophage lysin is added. The incorporated
lysin ensures early lysis of the starter culture and
accelerates cheese maturation by the release of a flavour
generating cocktail of starter enzymes.

An alternative is to encapsulate the lysin so that the
timing of its addition is not important. The
encapsulating agent dissolves after the cheese-making
process is complete thus not affecting the starter
bacteria before their role in acidification was complete.

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Key to Figures 1A and 1B

Figure 1A : A is a restriction map of ØvML3 which, at
arrow 1, is partially <u>AluI</u>-digested. Blunt-ended
fragments of 0.5-4.0 Kb are isolated, ligated with <u>EcoR</u>1
linkers and trimmed by digestion with <u>EcoR</u>1.

B is a map of vector lambda.gt 10, arrow 2 representing
<u>EcoR</u>1 digestion and dephosphorylation thereof to
lambda.gt 10 arms. At arrow 3, the product is ligated,
packaged <u>in vitro</u> into lambda phage and plated onto
<u>E.Coli</u> C6000 hfl⁻ for lysin production detection.

In step 4, the products of steps 1 and 3 are combined to
form a lysin-producing clone lambda gt.10-lysin 4, shown
at C. This is digested (5) with <u>EcoR</u>1 and ligated (6)
with <u>EcoR</u>1-digested and dephosphorylated vector pUC13,

shown at D. The product is transformed into E.Coli TB1 using agar with ampicillin, IPTG and Bluogal. White colonies producing lysin are selected.

Figure 1B: Clone pFI106 (E) is digested with EcoR1 and ligated (7) with EcoR1-digested lactic streptococcal vector pCK536 (shown at F) and (8) with EcoR1-digested vector pCK1 (at G). E.coli 1106 is transformed with the products of steps 7 and 8. chloramphenicol-resistant colonies are selected and two ampicillin-sensitive lysin producing clones are obtained, pFI136 (shown at H) and pFI137 (at I).

CLAIMS

1.   A formulation comprising the lysin of a Lactoccus bacteriophage or a variant of such a lysin and suitable for addition to cheese or to a cheese precursor mixture.

2.   A formulation according to claim 1 wherein the formulation is free of the said bacteriophage.

3.   A formulation according to Claim 1 or 2 wherein the bacteriophage is a prolate-headed bacteriophage.

4.   A formulation according to any of claims 1 to 3 wherein the bacteriophage is ϕvML3.

5.   A formulation according to any other of claims 1 to 4 which is encapsulated in capsules which release their contents at a predetermined time in a cheese-making process.

6.   A method of making cheese comprising lysing the starter culture with a lysin from a Lactococcus bacteriophage or a variant of such a lysin.

7.   A   method   according   to   Claim   6   wherein   the
bacteriophage is a prolate-headed bacteriophage.

8.   A. method   according   to   Claim   7   wherein   the
bacteriophage is φvML3.

9.   A method   according   to   any   one   of Claims   6   to   8
wherein   the   lysin   is   added   at   the   end   of   the   dairy
fermentation.

10.   A cheese made by a process according to any one of
Claims 6 to 9.

11.   A substantially pure preparation of the OvML3 lysin.

12.   A   φvML3   gene   or   coding   sequence   for   lysin,   in
isolation   from   the   genes   which   would   normally   lie
immediately upstream or immediately downstream of that
gene in φvML3.

13.   A coding sequence comprising the DNA coding sequence
given in Figure 7, or variants thereof.

14. A DNA construct comprising a coding sequence as in Claim 12 or 13 in an expression vehicle suitable for transformation of a microbial host.

15. A construct according to Claim 14 wherein the expression vehicle comprises an expression control region for the said coding sequence comprising the natural bacteriophage control region or a lactic streptococcal control region.

16. A microbial host transformed with a construct according to Claim 14 or 15 and capable of expressing the lysin coding sequence.

17. A polypeptide derived from the cultivation of a host according to Claim 16.
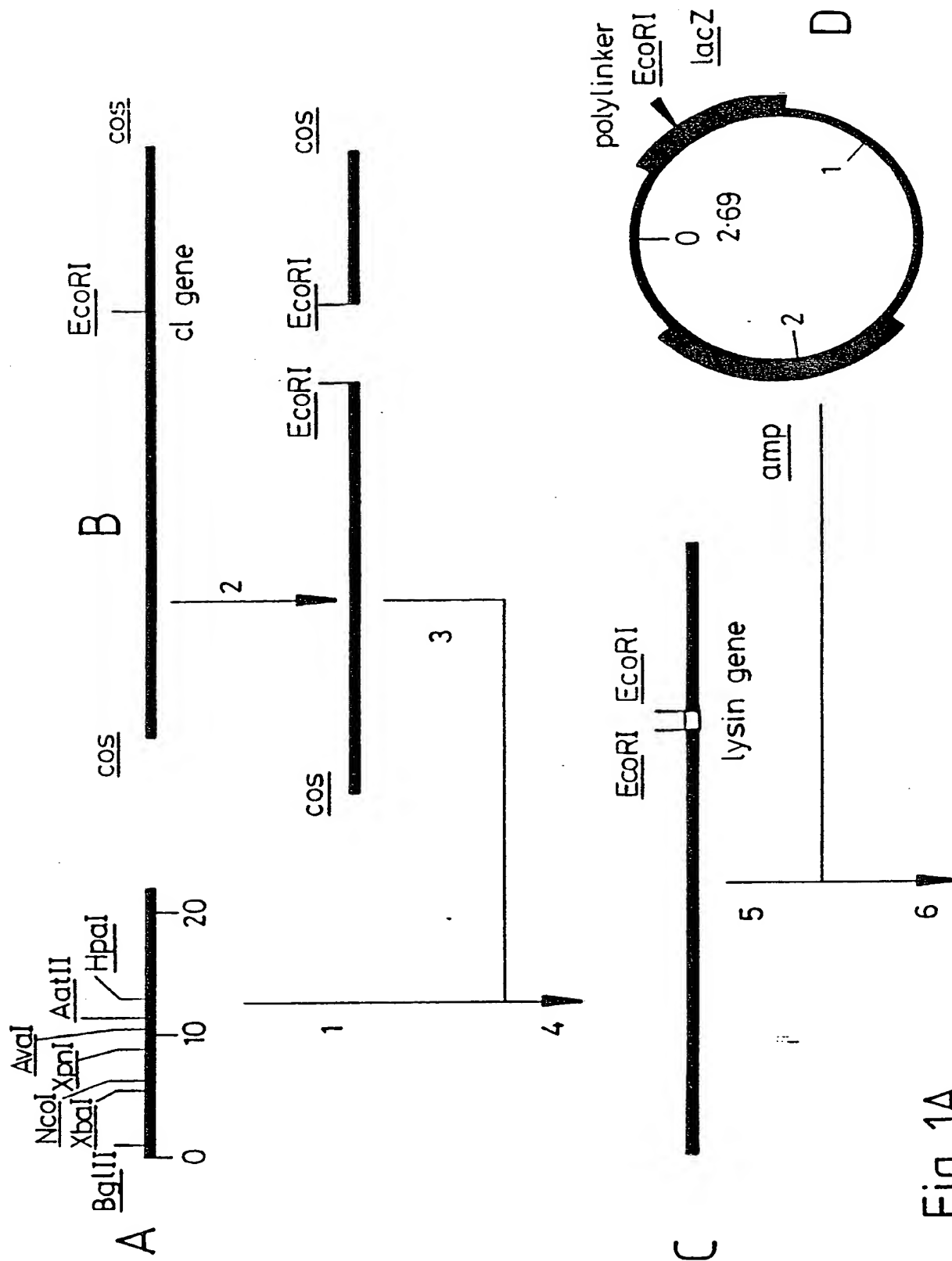
Fig. 1A

Fig.1B

Fig. 2
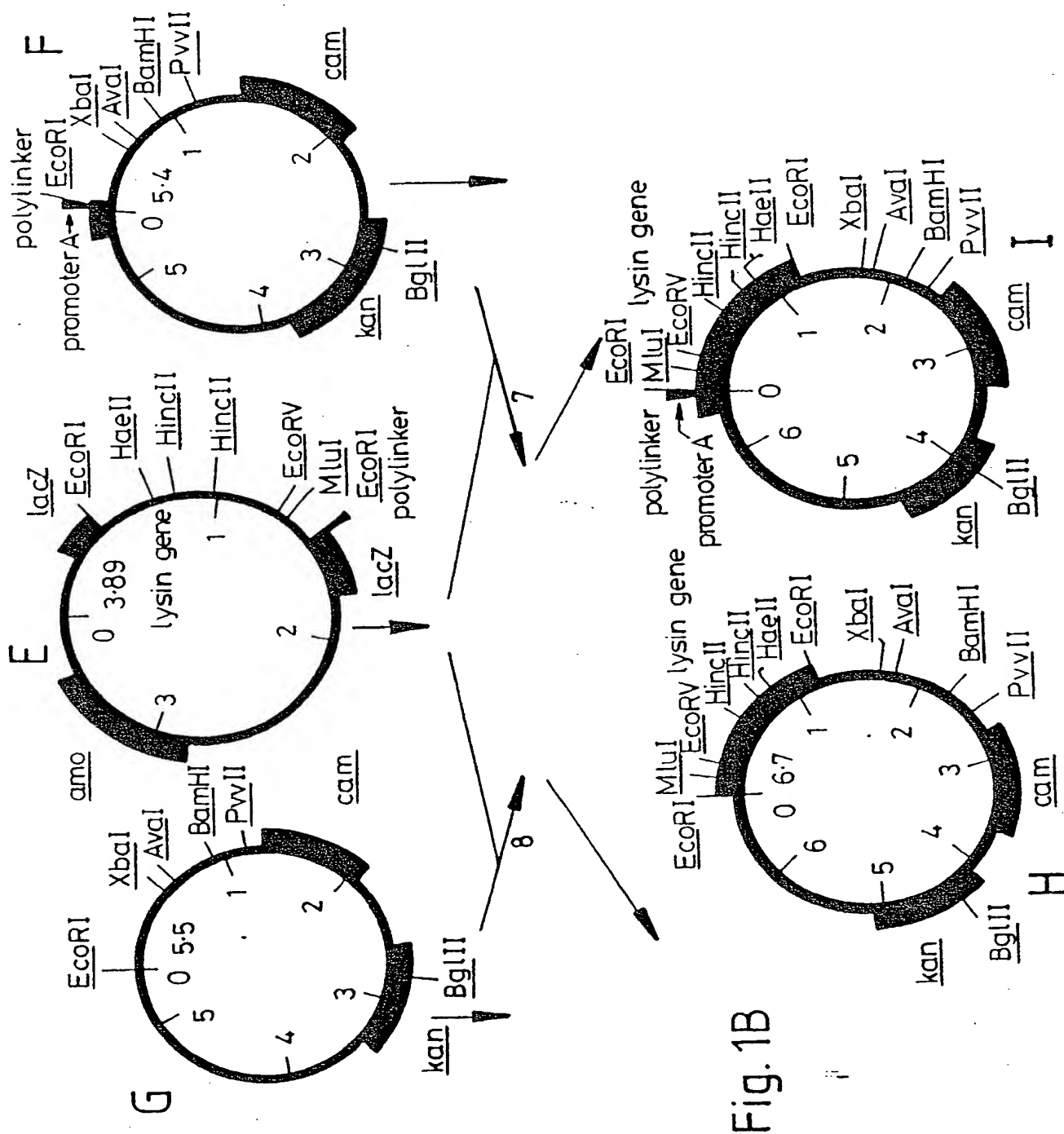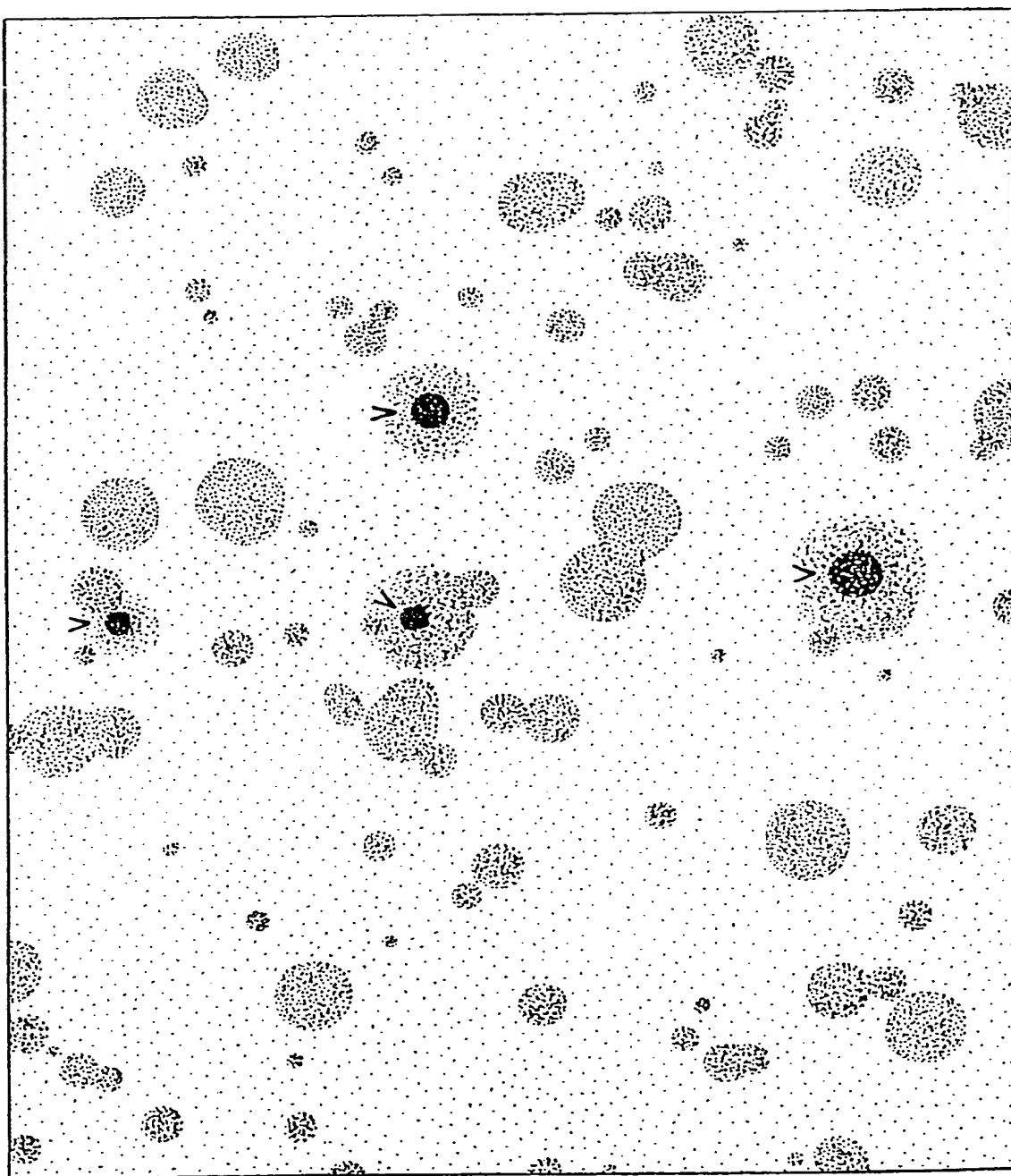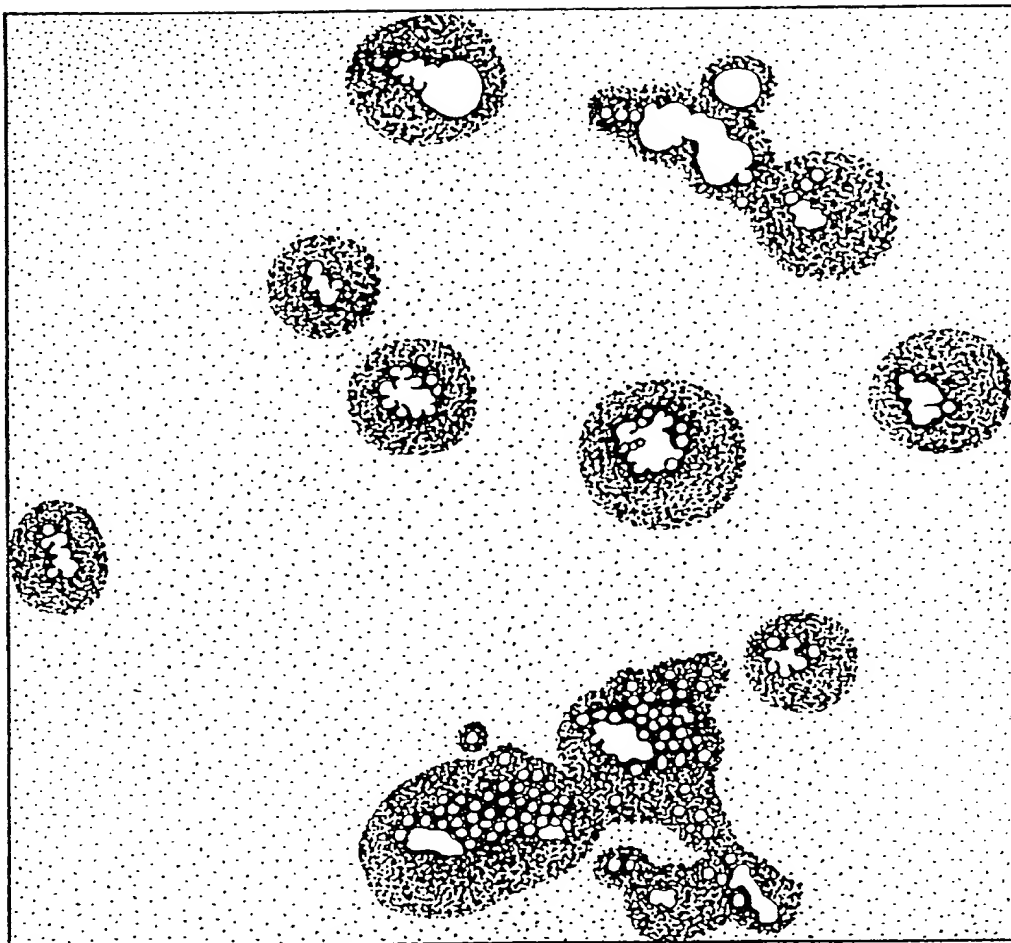
Fig. 3
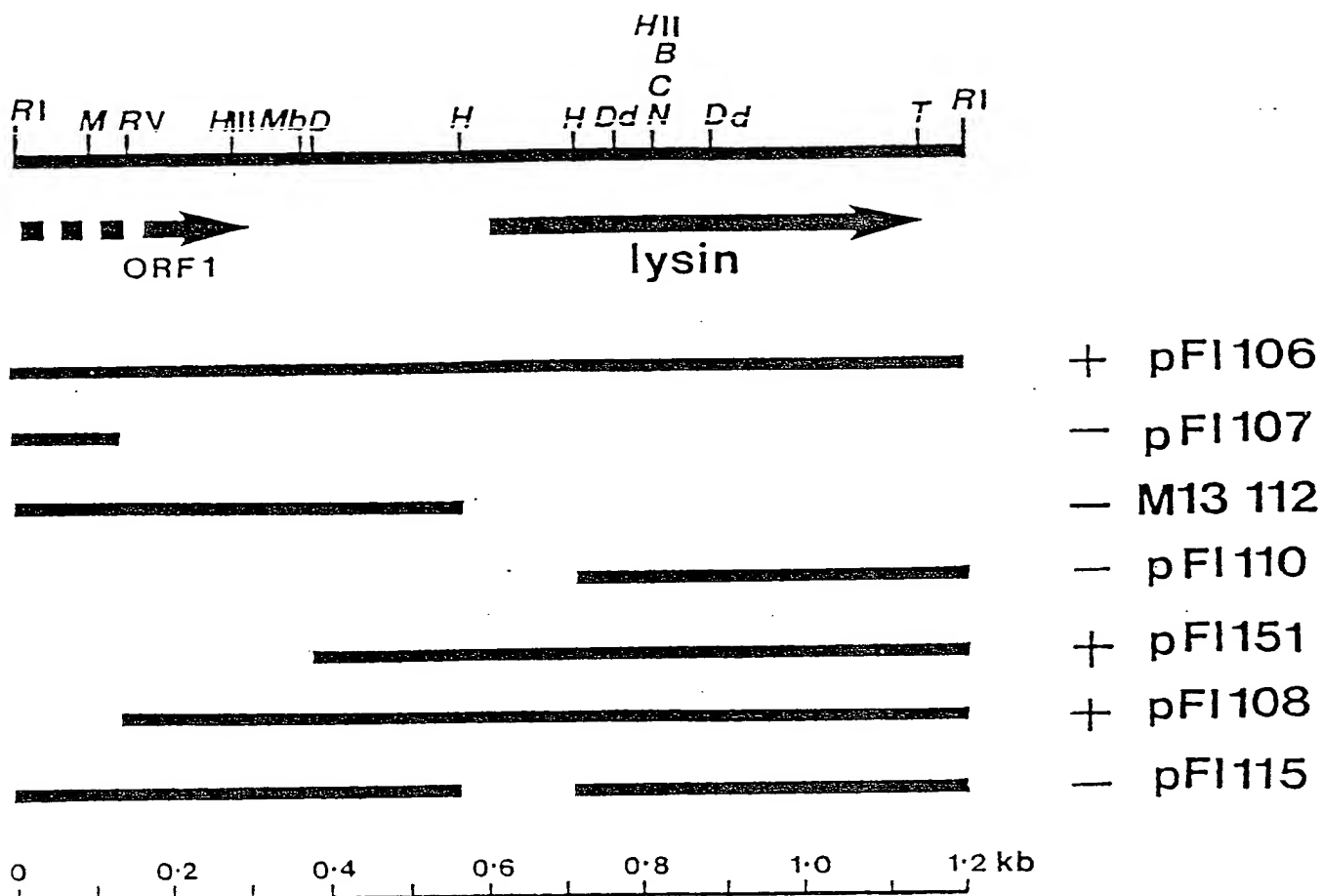
FIGURE 4

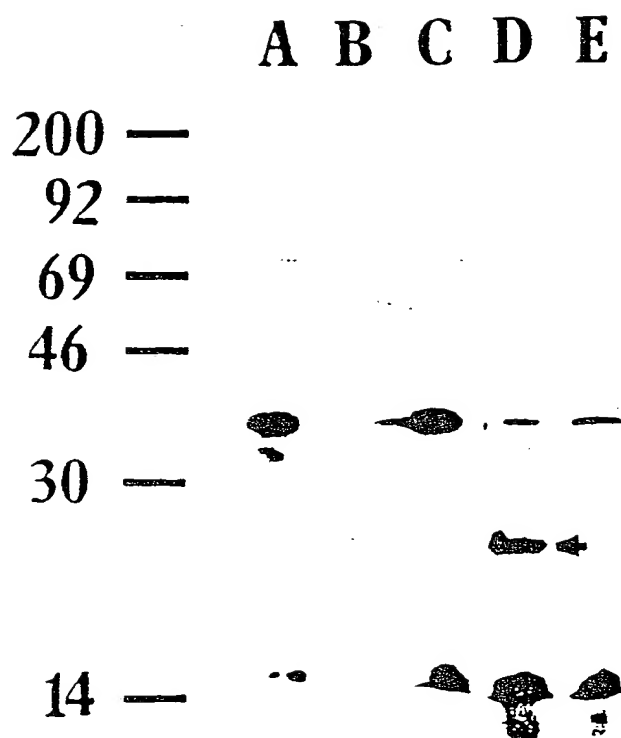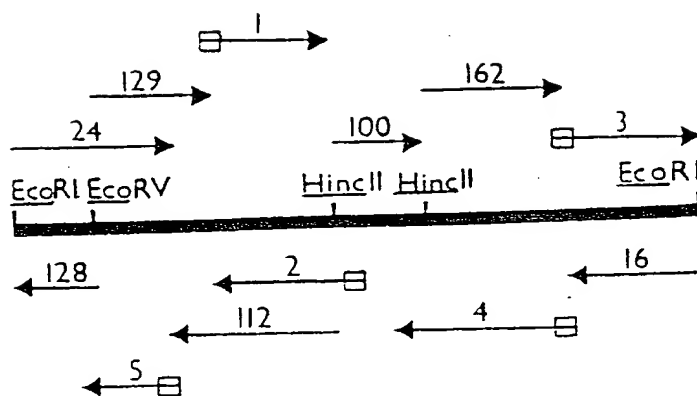FIGURE 5

FIGURE 6

```
        E   F   P   S   K   K   D   E   G   T   G   Y   A   F   R   K   D   G   Q   L   Y   V   G   S
GAATTCCCTAGTAAGAAAGACGAGGGAACTGGGTACGCCTTTAGAAAAGACGGACAATTATATGTCGGTTCC        72
EcoRI

        I   K   A   Y   N   A   K   K   N   A   W   E   R   T   F   D   I   V   N   A   I   K   D   I
ATTAAAGCATATAACGCAAAGAAAAACGCGTGGGAACGTACTTTTGACATTGTGAACGCAATTAAAGATATC        144
                                                                    EcoRV

        I   D   E   F   D   L   K   D   Y   H  [M]  A   I   E   T   P   I  [M]  G   R   N   R   K   H
ATAGATGAGTTTGACTTAAAAGACTATCACATGGCTATTGAAACGCCTATCATGGGTAGAAACAGAAAGCAC        216

        S   I   T   L   A   N   C   N   G   Y   F   I   G   A   I   D   G   L   V   N   G   L   Y   F
AGTATCACATTGGCTAATTGTAACGGTTATTTTATCGGTGCTATTGACGGTCTAGTAAATGGCCTATACTTT        288

        Y
TATTGATAACTCTAAATGGTGTAGCTATCATTTAATTTCAGGCAAACGAGAACAACGAAAAGAAGAAAGTTT        360
         5                        1

GGAACTTTTAAAACAGACTGGACTTGTTCCGCTTGATTGTAAAGATGATAACATGGCTGACGCTTATAACAT        432
    DraI

TTTGACATATTGCGAACACTTGGGTTAGTTGTTCCCTTATAAAAAAACAATAATAATAATTGGAGGTGGTAA        504

TATAAAAGTATCACAAAACGGTTTGAACTTGATTAAAGAGT TTGAGG GTTGTAGGTTGAC TGC TTATAA ACC        576
                                             HincII

                M   Y   T   I   G   W   G   H   Y   G   V   T   A   G   T   T   W   T   Q
TGTACCGT GGGAA CAAATGTACACTATCGGTTGGGGTCATTATGGAGTGACAGCAGGTACAACATGGACACA        648
                                                  2

        A   Q   A   D   S   Q   L   E   I   D   I   N   N   K   Y   A   P   M   V   D   A   Y   V   K
AGCTCAAGCAGATAGCCAGCTAGAGATTGACATCAATAATAAGTATGCACCTATGGTTGACGCTTACGTAAAA        720
                                                        HincII

        G   K   A   N   Q   N   E   F   D   A   L   V   S   L   A   Y   N   C   G   N   V   F   V   A
AGGCAAAGCAAATCAAAATGAGTTTGACGCCTTAGTTTCATTGGCTTATAACTGTGGTAATGTTTTCGTTGC        792

        D   G   W   A   P   F   S   H   A   Y   C   A   S   M   I   P   K   Y   R   N   A   G   G   Q
TGACGGTTGGGCGCCTTTCTCACATGCTTATTGTGCTTCAATGATACCGAAGTATCGTAATGCAGGCGGTCA        864

        V   L   Q   G   L   V   R   R   R   Q   A   E   L   N   L   F   N   K   P   V   S   S   N   S
AGTCTTACAAGGCTTAGTAAGACGCAGACAGGCAGAGCTTAACTTATTTAATAAACCAGTATCAAGTAATTC        936
                                       3

        N   Q   N   N   Q   T   G   G   M   I   K   M   Y   L   I   I   G   L   D   N   S   G   K   A
AAACCAAAACAATCAAACAGGAGGAATGATAAAAATGTACCTTATTATAGGACTAGATAATTCAGGTAAAGC        1008
                                                  4

        K   H   W   Y   V   S   D   G   V   S   V   R   H   V   R   T   I   R   M   L   E   N   Y   Q
TAAACATTGGTATGTTTCTGACGGTGTAAGTGTTCGTCATGTTCGTACAATTCGTATGTTGGAAAACTATCA        1080

        N   K   W   A   K   L   N   L   P   V   D   T   N   V   Y   C   R   N   R   S   R   V   W   T
AAACAAATGGGCTAAACTTAACTTGCCAGTTGATACCAATGTTTATTGCAGAAATCGAAGCAGAGTTTGGAC        1152

GTAAGATTGACATGGCTTCAGGAGAAGTGAAATAGGAGGAAGTGAATGAGGGAATC        1209
                                    EcoRI
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FIGURE 7

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: C 12 N 9/14, A 23 C 19/032, C 12 N 15/55, C 12 N 1/21,
C 12 N 15/74

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC⁵	C 12 N, A 23 C

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category *	Citation of Document, ¹¹ with Indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	J. Dairy Sci., vol. 70, 1987, J.M. Feirtag et al.: "Isolation of Streptococcus lactis C2 mutants selected for temperature sensitivity and potential use in cheese manufacture", pages 1773-1778 see the whole article	1-3
Y	J. Dairy Research, vol. 52, no. 1, 1985, W.M.A. MULLAN et al.: "Lysin production by ⌀C2(W), a prolate phage for Streptococcus lactis C2", pages 113-121 see the whole article	1-3
Y	J. Dairy Research, vol. 52, no. 1, 1985, W.M.A. MULLAN et al.: "Partial purification and some properties of ⌀C2(W) lysin, a lytic enzyme produced by phage-infected cells of Streptococcus lactis C2", pages 123-138 see page 135-137, "discussion"	1-3

* Special categories of cited documents: ¹⁰
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the International filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th October 1989	27. 11. 89

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document. with indication. where appropriate. of the relevant passages	Relevant to Claim No
A	Journal of Virology, vol. 61, no. 8, August 1987, American Society for Microbiology, J.L. Garcia et al.: "Cloning, purification, and biochemical characterization of the pneumococcal bacteriophage Cp-1 lysin", pages 2573-2580 see abstract	12-17
A	Journal of Bacteriology, vol. 145, no. 1, January 1981, J.L. Raina: "Purification of Streptococcus group C bacteriophage lysin", pages 661-663 see the whole article	1-3,11
A	Chemical Abstracts, vol. 63, 1965, (Columbus, Ohio, US), J.D. Oram et al.: "Phage-associated lysins affecting group N and group D streptococci", see column 13631a, & J. Gen. Microbiol. 40(1), 57-70 (1965) see abstract cited in the application	1-4,11
A	Chemical Abstracts, vol. 73, 1970, (Columbus, Ohio, US), J.M. Erskine: "Effect of spermine on host-cell lysis and reproduction by a lactic streptococcal bacteriophage", see page 100, abstract no. 1175e,. & Appl. Microbiol. 1970, 19(4), 638-42	
T	Molecular General Genetics, vol. 218, August 1989, C. Shearman et al.: "Cloning and DNA sequence analysis of a Lactococcus bacteriophage lysin gene", pages 214-221 see the whole article	

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